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Recent Advances In Bone Growth And Nutrition

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Recent Advances in Bone Growth and Nutrition

By W. F. Neuman, Ph.D. and M. W. Neuman, Ph.D.

THE CHEMISTRY AND BIOCHEMISTRY OF BONE

The purpose of this review is to summarize the newer findings concerning the mechanisms of bone growth, particularly the calcification mechanism. It also is hoped that this summary will place in fresh perspective both the old and new knowledge of the important actions of vitamin A, the B-complex vitamins, ascorbic acid, vitamin D and other nutritional influences on bone.

Man has a tremendous mass of mineral reserves in his skeleton: in the adult it amounts to some 1,200 grams of calcium, 1,500 grams of phosphate, with carbonate, citrate, magnesium, sodium, fluoride and traces of other elements intermixed. This mineral reservoir is in the form of "zillions" of tiny crystals located in and around sheets and bundles of collagen fibers. Its approximate proportions, by weight, are: 60% inorganic substances, 20% collagen and 15% water (free form) with traces of mucopolysaccharides and other proteins. Cells, too, are present, but these comprise not more than 5% of the total volume.

This great mass of crystals is *extracellular*. It, therefore, is presumed to be in contact with the body fluids and equilibrating continuously with them. In some parts of bone, however, crystals are forming while in others, crystals are dissolving; this occurs with relatively constant levels of calcium phosphate in the blood. Most of these crystals, however, appear to be reasonably stable in the bulk of the skeleton despite widely varying levels of calcium and phosphate in various disease states. Nevertheless, there are disease states which provide examples of every conceivable kind of variability. (See Table 1.)

TABLE I

CALCIUM-PHOSPORUS SERUM LEVELS UNDER VARIOUS CONDITIONS

Normal	Ca	P↑↓
Hyperparathyroid	Ca↑	P↓
Hypoparathyroid	Ca↓	P↑
Calcium Infusion	Ca↑	P↑
Steatorrhea	Ca↓	P↓
Experimental Rickets	Ca	P↓
Experimental Rickets	Ca↓	P

THE SOLUBILITY DILEMMA OF BONE MINERAL

To the chemist, the situation of bone mineral has been a fascinating problem in solubility. Here is a proven case of solid phase formation, stability and dis-

solution; all occurring in an aqueous medium which usually is rather fixed in composition, but also may be quite variable. This conundrum stood, unresolved for years, like a colossus guarding the gates to further understanding.

Now, at long last, the puzzle seems to be solved for we have the answers to these three questions:

1. Is serum undersaturated with respect to calcium and phosphate?
2. Is serum exactly saturated with respect to calcium and phosphate?
3. Is serum supersaturated with respect to calcium and phosphate?

The answers are, respectively, "yes," "no," and "yes."

Actually, these three answers were established by earlier workers many years ago,[5, 8, 24, 26, 44, 56, 68, 77, 106] but the implications were so confusing that those investigators turned to other, more profitable, fields of endeavor. Approaching the problem with a viewpoint ingrained by years of training in chemistry, the pioneers were frustrated on finding an aqueous solution simultaneously both undersaturated and supersaturated. This was contrary to every chemical expectation: Thus, into the resulting conceptual vacuum moved controversies and unfounded hypotheses.

Resolution of the solubility dilemma did not involve a suspension or revocation of physicochemical principles. Rather, it required only that *two different solid phases* be considered: one, the bone mineral itself, governing dissolution, and two, secondary calcium phosphate ($CaHPO_4 \cdot 2H_2O$), governing precipitation. In substance, this system was proposed in 1928,[107] but it never gained wide acceptance. Today, it seems simple enough and one might wonder why. One answer is that secondary calcium phosphate is only an evanescent intermediate, a solid phase never found to exist under physiological conditions.[119] This explains its absence in calcified structures.[111] The solubility product, K_{sp} $CaHPO_4$ however, does represent the upper stability limit of solutions containing calcium and phosphate[119] and, therefore, of serum. Another answer is that most investigators, in the habit of using Occam's Principle, thought of bone mineral as a single chemical entity with a fixed or normal solubility. It is now recognized that bone mineral is variable in composition. Far from being a single chemical entity, the solid phase of bone belongs to a family of chemical compounds which has a common spatial structure undisturbed by wide variations in chemical composition.[88] The prototype of this structurally isomorphic series is hydroxy apatite, $Ca_{10}(PO_4)_6(OH)_2$.

Besides variability in the calcium and phosphate content inherent in the apatite structure itself, additional variability is made possible by surface reactions. Because the crystals are so small, colloidal in size, many foreign ions are found associated with the solid phase, bound by exchange-adsorption and other surface effects.[88] These are not trace impurities; they are of quantitative importance since one-fourth to one-third of the total ions present are located in the surface or at the surface-solution interface.[88] This means that the composition of the mineral changes on exposure to different solutions. In fact, the solid reflects, to a degree, the composition of the medium in which it is placed.

It is helpful first to consider the solubility properties of the aqueous apatite system quite apart from the more complicated system obtained *in vivo* — the organic elements of bone and of extracellular fluid. Even the simpler system is complex enough, for "apatite" was aptly named. The term is derived from the Greek meaning, "the deceiver."

Calcium Phosphates in Simple Solutions

Even in water, because of variations rendered, it is extremely difficult to get reproducible solubility data. Far from being a constant, the observed solubility value depends on the past history of the solid, the solid:solution ratio, and the changing composition of the water as the solid dissolves. In fact, the solubility of bone minerals cannot be described by any simple ion product. All conceivable ion products show a variability covering several powers of ten.[76]

Secondary calcium phosphate, on the other hand, is a well-behaved substance exhibiting a thermodynamic K_{sp} and forming large, beautiful crystals of defined composition. The difficulty is that secondary calcium phosphate is stable only in acid or slightly acid solutions. At physiological pH, crystals of this substance disintegrate and hydrolyze spontaneously to the more basic apatite-series of materials.[119]

Another interesting property of the aqueous calcium phosphate system is that concerning solid phase formation. The apatites, containing approximately eighteen ions in the smallest unit of structure, are much too complicated to be formed directly by simultaneous collisions of ions in solution. Only by the step-wise addition of ions at some preformed surface can crystals of apatite grow. In pure solutions, there is no preformed surface. It follows, then, that solutions of calcium and phosphate are stable until some solid structure simpler than that of apatite forms. Secondary calcium phosphate, though more soluble, serves as such a seeding substance. Its formation requires a collision of only two ions and some solvent molecules. As soon as it forms at physiological pH, of course, it begins to disintegrate; the aggregates, however, can furnish the surface needed for the step-wise growth of apatite crystals. Thus, at physiological pH, precipitates initially exhibit a Ca:P ratio of unity, corresponding to $CaHPO_4 \cdot 2H_2O$. Within minutes after precipitation, the ratio starts to rise and the x-ray diffraction pattern suggests an amorphous structure. Within an hour, the Ca:P ratio approaches 1.6, theoretical for apatite; the apatite pattern is given on examination by x-ray.[119] This instability, coupled with the undersaturation of normal serum, no doubt accounts for the failure of investigators ever to find $CaHPO_4 \cdot 2H_2O$ in animals, despite many determined efforts.

That apatite sets the range of solubilities in *dissolution* experiments while secondary calcium phosphate sets the limit of stability of solutions in *precipitation* experiments is now established. This constitutes the resolution of the old solubility dilemma; however, still more complications are encountered in turning to the systems operative *in vivo*.

Calcium and Phosphate Levels In Vivo

First, we must define the normal levels of calcium and phosphate ($HPO_4^=$) ions found in human serum. Lacking a direct method for the determination of free calcium ion, it has been necessary to deduce its concentration by inference. By ultrafiltration techniques, it is possible to estimate that some 35% of the 10 mg% of calcium normally found in serum is bound to protein.[126] Of the filtrable calcium, less than 20% is in the complex form associated with citrate, carbonate and phosphate. About 5.3 mg% is free and ionic.[89] The phosphate system, simpler than calcium chemically, is more variable physiologically. Total inorganic phosphate can vary from 2 to 6 mg% depending on the age and metabolic state of the individual. An average adult fasting level would be a little over 3 mg%. This gives a normal serum product, Ca x P inorganic of about $20(mg\%)^2$ with values as low as 10 or as high as 30 not uncommon pathologically.* (See Figure 1).

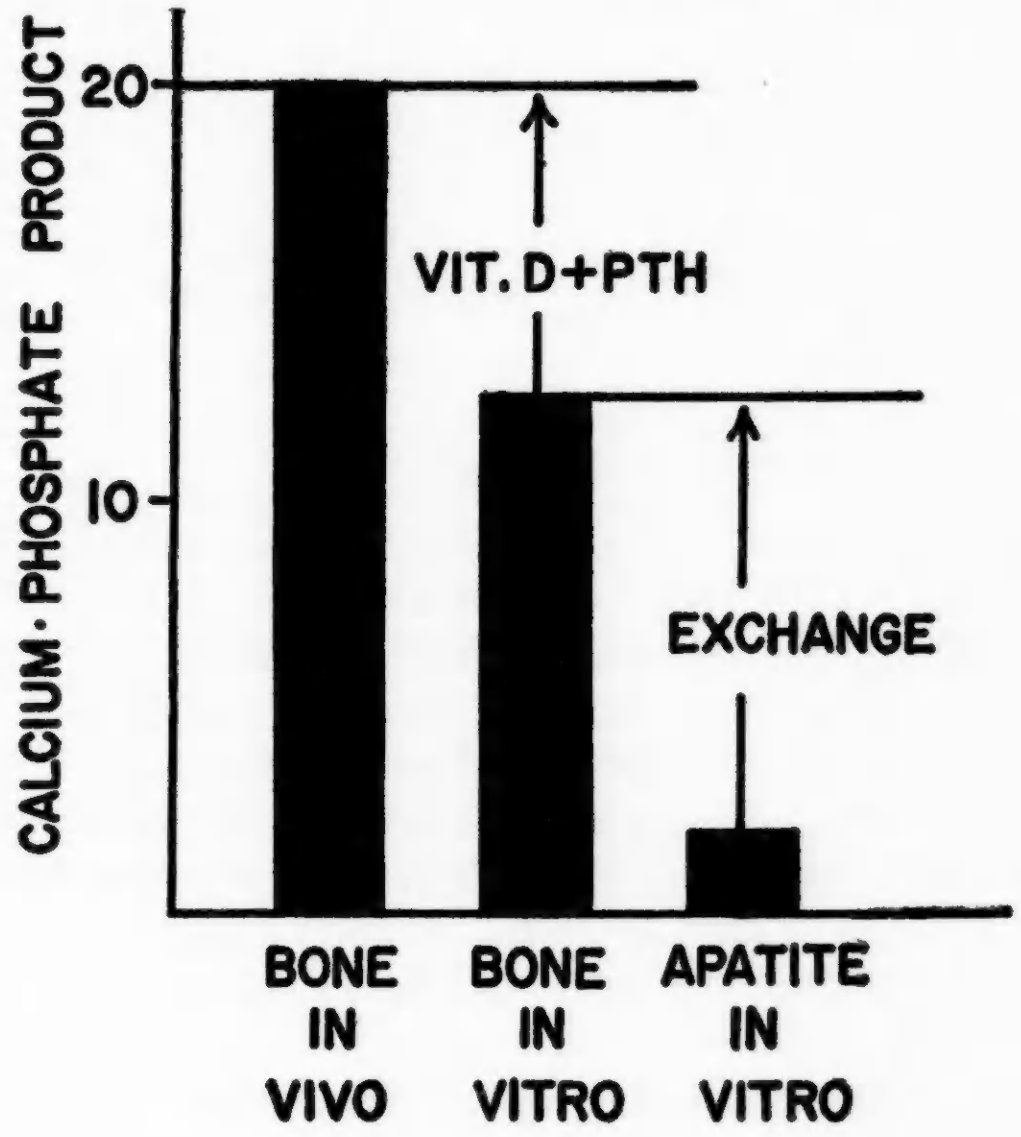
The range of ion products in serum may now be compared with the products given on *dissolution* of bone mineral and with the products required to cause precipitation of calcium phosphate from solution. From the problems of variability of solubility already discussed, we can expect not one constant but rather a wide range of values. For practical purposes, however, these problems of variability can be ignored. Starting at the right of Figure 1, pure hydroxy apatites are extremely insoluble giving dissolution products, Ca x P_i, of one-tenth or less of those of normal serum. Next, ashed bone, powdered dead bone and similar "impure" apatites are somewhat more soluble, particularly in solutions such as serum containing bicarbonates and citrates which undergo surface reactions with the apatite. Even these preparations at pH 7.4 give dissolution products, Ca x P_i, of only roughly ½ that of normal serum. Clearly then, serum is supersaturated with respect to bone mineral. Later we shall return to the problems raised by this stubborn fact: maintaining normal serum levels of calcium and phosphate above the natural solubility of the mineral phase, and dissolving crystals *in vivo* in areas where resorption is necessary physiologically.

Turning to the *formation* of solid phase we again find a *range* of products, Ca x P_i, which spontaneously will precipitate. But again, the practical case is clear. Spontaneous precipitation never occurs below the K_{sp} of $CaHPO_4$, corresponding to a product of about $35(mg\%)^2$ or nearly double that of normal serum and well above the range of normal variation.[119] Solutions having products in the range 35 to $60(mg\%)^2$ may, under certain conditions, be stable for

*To be physicochemically correct, these concentrations should be expressed in terms of ion activities. The Ca^{++} activity would be 36% of its concentration. The concentration of $HPO_4^=$ is 81% of the total inorganic phosphate and its activity would be 37% of this. However, in serum the pH, ionic strength, and specific calcium complexers are fixed and the crude ion products are accurate (though higher than the activity product by a constant, 1.86×10^{-8}). Thus, the activity product for normal serum, $\alpha Ca^{++} \cdot \alpha HPO_4^=$, is slightly over 1×10^{-7}. This holds true for simulated serum solutions as long as the pH, ionic strength, and calcium complexers are equivalent to those in serum. Otherwise, activity corrections *cannot be ignored* if a comparison with serum is to be made.[89]

protracted periods before crystals form. This is a region of *metastability*, a condition familiar to chemistry students. In such a solution, crystals can be caused to form if the beaker is scratched or if seed crystals are added. These solubility principles must operate in the calcification process.

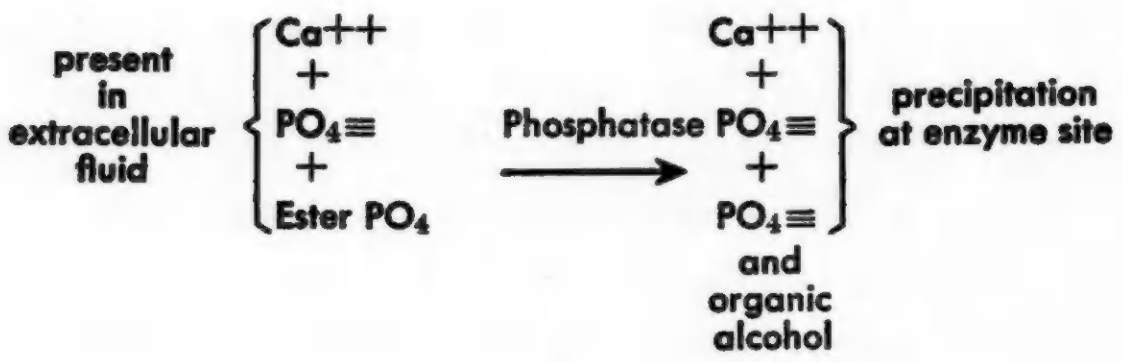
Figure 1. A diagrammatic summary of experience with the solubility of bone mineral and its prototype. See text for details.



THE MECHANISMS OF CALCIFICATION

Historical Background

The newer views of calcification now take cognizance of the high degree of undersaturation of normal serum with respect to solid phase *formation*. Older views, for the most part disguised or undisguised modifications of Robison's original postulates,[103] erroneously presumed serum to be just saturated with respect to bone mineral; they further assumed that a slight local elevation of the phosphate level would bring about solid formation at the desired sites. Robison was led to elaborate his hypotheses by observations made in purifying the enzyme, phosphatase. Employing alkaline earth salts of esterified phosphate compounds, he noted phosphate precipitates whenever the enzyme was present. A careful search demonstrated phosphatase to be present at all calcifying sites and the case seemed complete for a mechanism which may be summarized as:



Unfortunately, besides the errors in the assumed saturation of body fluids, the necessary substrate-ester could not be demonstrated. To overcome this defect, several workers postulated a cellular origin for the missing ester and a vaguely-defined "Second Mechanism"[104] was born in the literature. Today the Robison Scheme has fallen into disrepute, though the function of phosphatase has not yet been clarified. Possible functions of this undoubtedly important enzyme will be discussed later.

Seeding of Crystallization by Collagen

The first clue to an alternative mechanism more consistent with solubility considerations came from studies of the stability of solutions of calcium and phosphate *in vitro*. Crystals of calcium phosphate came out of otherwise stable solutions when powdered apatites or bone mineral were added.[119] This was not surprising because of the low solubility of bone mineral itself. The surprise was that purified, reconstituted collagen fibers also induced solid phase formation. Other proteinaceous substances, fibrin for example, were ineffective.[119] Since then, several laboratories have successfully crystallized calcium phosphate from otherwise stable solutions by means of collagen preparations.[2, 41, 70, 118]

The solid which forms under such circumstances has been shown to be, like bone mineral, an apatite.[46] Further, this seeding process has been shown to be exquisitely specific. In recent years, collagen has itself been crystallized in a number of forms identifiable in the electron microscope.[46] Of these various forms, only the one which occurs naturally in bone and connective tissue, that with regular banding at about 640 A°, is effective in seeding solutions of calcium and phosphate.[40] Finally, the appearance of beginning mineralization *in vitro* appears to be morphologically identical with slightly mineralized collagen fibers taken from forming bone.[30, 40]

One defect of these studies has been their quantitative relation to the *in vivo* system. Where conditions were reported, these collagen preparations were effective only in solutions having products, Ca x P_i, above the physiological range. Recently, however, demineralized dentin preparations [113] and a purified collagen[31] have brought about mineral formation at products of 20(mg%)^2 and below. (See Figure 1.) Thus, the last remaining criticism of the collagen-induction mechanism seems to have been removed.

There still remain, however, many details to be elaborated by further research. While collagen-induction of mineral seems established in principle, there is no

information concerning the molecular properties of the collagen involved. Apart from the importance of the crystallographic form of the collagen mentioned earlier, there is some evidence that the ε-amino groups of lysine may participate.[112] The role of the mucopolysaccarides is not at all clear. Some think that mucopolysaccharides are beneficial,[3] others that mucopolysaccharides are inhibitory to the seeding-process.[40] That these carbohydrates are important no one doubts. In the animal, some collagenous tissues calcify while others do not, and changes in the staining properties of the associated carbohydrates are consistently observed in calcifying regions.

Possible explanations of this tissue-specificity, other than the state of the mucopolysaccharides and the constitution of collagen, may be mentioned. Local conditions might easily be crucial. The osteogenic cells may elaborate metabolites, regulate pH or other variables which may improve or block nucleation. Another intriguing possibility concerns the enzyme, phosphatase. Small concentrations of ester-phosphates have been shown to inhibit the growth of apatite crystals in inorganic solutions.[86] More recently, ester-phosphates have been shown to inhibit seeding by collagen fibers and there seems to be present some substance, of similar action, in serum ultrafiltrates.[31, 113] Could it be that phosphatase acts locally to destroy a crystal-seeding poison (ester-phosphate) and thus permit crystal formation to occur only in the environment immediately adjacent to the enzyme itself?

Perhaps all these factors are necessary. It easily could be that, for crystals to form *de novo* in the animal, there is required:

1. a special collagen of specific crystallographic form and/or chemical reactivity for effective seeding,
2. a special mucopolysaccharide or mucopolysaccharides in a specific state of aggregation,
3. the enzyme phosphatase to destroy the postulated crystal-poisoning esters, and
4. a special environment (pH?) controlled by the adjacent cells.

In any event, the importance of the cell in calcification cannot be questioned. It must provide, minimally, a matrix which is calcifiable locally while the organism as a whole must provide a *milieu* which is adequate in mineral content and not overrich in crystal-inhibitors.

THE REGULATION OF THE INTERNAL MILIEU

The maintenance of an internal milieu favorable to bone formation is a much more complicated matter than that of merely eating the proper foodstuffs. Particularly involved are three organ systems (intestine, kidney and bone), at least one hormone (parathyroid hormone) and at least one vitamin (vitamin D). The importance of these organs and agents in maintaining calcium homeostasis (a term which also implies phosphate homeostasis) has long been recognized. Of late, however, their interrelationships have been considerably clarified as a

result of the advance in our understanding of the role of bone itself in the homeostatic machinery. As with the mechanism of calcification, the impediment to progress was the confusion extant concerning solubilities.

Regulation by Bone

Recognizing as we now do that serum normally is highly supersaturated with respect to bone mineral, the problem is seen in clear perspective. There is still, of course, the problem of which comes first, the chicken or the egg. In this case the question may be posed thus:

1. Given a supersaturated serum, what is to prevent bone from mineralizing rock-solid, so filled with crystals that even diffusion is impossible? Or,
2. Given a great volume of highly insoluble crystals, what is to prevent the serum levels of calcium and phosphate from falling to near-solubility levels?

These parallel questions have parallel answers:

1. Bone does tend to mineralize rock-solid, but the bone cells modify this, in part, by secreting acid metabolites favoring dissolution.
2. Serum levels do tend to fall, but the acid secretions of the bone cells offset this tendency.

Here is the situation in a nutshell: apatites give dissolution products, Ca x P_i of about one-tenth that of serum. Ashed bone or powdered dead bone preparations give dissolution products, Ca x P_i, about one-half that of the serum. Living bone in culture gives higher dissolution products, Ca x P_i, tending to be somewhat low but approaching those of serum. (See Figure 1.) Clearly, it is the presence of cells which accounts for the apparent high dissolution products *in vivo,* but how can cells affect a mineral:solution equilibrium *extracellularly?* The answer to this question is found only by considering the nature of the bone cell and the fluid exchange within bone. If serum is supersaturated with respect to bone mineral it does not necessarily follow that the fluids in bone are also supersaturated. The two fluids are not necessarily equivalent in composition.

It is said that, except for bone, the capillaries of the tissues are never separated by more than a distance equivalent to two cells. In compact bone, however, the Haversian blood vessels are separated by a distance of many cells and the exchange of nutrients and waste products must be made, for the most part, by diffusion along the tiny canaliculae, the fine interconnecting processes of the bone cells themselves. Compared to other tissues, the skeleton is a diffusion swamp where localized gradients in the metabolic fuels and the end products of cells would be expected on the basis of logic alone.

It has been suggested, by inference, that the pH of the bone fluids may be lower than that of serum.[94] This is probably true, but, experimentally, pH is extremely difficult to measure accurately in a system such as bone. In any event, it has been shown that bone cells do produce acids both *in vivo* [14, 29, 87] and *in vitro.*[23, 42, 64] Furthermore, it has been demonstrated that the production of one acid, citrate, in bone is directly related to parathyroid status.[79] Parathyroid-

ectomy, which leads to dense bones and diminished resorption, abolished citrate output by bone. On the other hand, injection of the hormone which rarefies bone and increases resorption, raised the output of citrate by bone above the normally positive values found in blood leaving bone as compared to those in arterial blood. Lactate production by bone cells has also been shown to be directly related to parathormone level, both *in vivo* [79] and *in vitro*.[23]

The details are not yet worked out, but the general principle seems clear. Bone mineral can be dissolved by citrate and other acids as well as by other cell metabolites. A given ion product, Ca x P_i, which may be highly supersaturated in circulating serum where the pH equals 7.4 and [citrate] equals 1 x 10-4M may actually be undersaturated in the bone fluids where we may make a rough estimate that the pH equals 6.9, and [citrate] equals 5 x 10-4M. The serum which is indeed supersaturated is not the fluid which is in contact with the bone crystals. It represents, rather, the overall average ion product, Ca x P_i, resulting from the total, skeletal cellular activity which by regulation of its acid production is eroding the bone substance in some areas, is in balance in other areas and is laying down crystals in still other areas.

Despite the fact that serum levels of calcium and phosphate represent the variables which call homeostatic mechanisms into action (the stimulation of parathyroid secretion by hypocalcemia may be cited), it is the skeleton itself which dominates the composition of the internal *milieu*. The mineral content of skeleton is gigantic compared to the mineral content of serum. An altered Ca:P or Ca x P in serum just cannot occur without some concurrent disturbance in the balance of cellular activity in the skeleton itself. The disturbance may be direct, resulting from abnormal parathyroid status, for example, or it may be indirect, resulting from mineral loss through a deranged kidney function. Nonetheless, the truism still holds. Only by a change in the balance of cellular activity in the skeleton can the apparent skeleton:blood equilibrium be altered.

Regulation by the Kidney

Despite the dominance of the enormous mineral reserves of the skeleton in calcium homeostasis, the kidney, too, plays its usual, essential role. This organ was designed by evolutionary selection as a means of conserving water, thus freeing animal forms from dependence on the Mother Sea. Yet, the kidney conserves calcium even more efficiently than water. In fact, one gains the impression that *all* of the calcium ion, filtered at the glomerulus, is reabsorbed while only calcium in complex form passes out into the urine.[13, 14, 72] The reabsorption appears to be an active process, occurring in the distal tubule,[57, 31] different from that involving the alkali metals, but one requiring cellular energy. With over 99% reabsorption occurring normally and less than 1% excretion, a very slight change in reabsorption represents a large change in excretion (99.2:0.8, 99.5:0.5, 99.8:0.2). Overall, however, the renal excretion seems less a matter of regulation than one of conservation.[58] Were it not for the magnificently efficient reabsorption of calcium by the kidney, enormous quantities of calcium would need to be

absorbed daily from the diet. Thus, the dependence of dietary intake of calcium upon normal renal function is easily seen.

Conservation of phosphate, too, is dependent on normal renal function. At low filtered loads, the tubular reabsorption of phosphate, like that of calcium, approaches 100 per cent. With increased glomerular flow or elevated serum phosphate levels, it is possible to demonstrate a T_{max}, or maximal rate of tubular transport (presumably in the proximal tubule) with the excess phosphate spilling into the urine. This maximal rate is about 0.15m M/min/M^2 in the adult dog and man.[97] The T_{max} seems to be relatively constant; urinary excretion of phosphate, is mostly a function of plasma level and glomerular filtration rate. In some species, an actual secretion of phosphate by the tubules has been reported.[92] However, the T_{max} can easily be interpreted as an excretion threshold, above which essentially all filtered phosphate appears in the urine. Viewed in this way, the threshold appears to be under physiological control, responding to drugs (lowered by pentobarbital) and the phosphate load[33] (lowered by an increased load). The threshold concept has also been used to explain the "on and off" phosphaturic actions (notoriously nonreproducible) of acute administration of parathyroid hormone which seems effective only when the threshold is low. Prolonged administration of the hormone lowers the threshold.

Renal excretion of phosphate is further complicated by intra-extracellular shifts derived from changes in the systemic glucose levels, hormone levels, and from the generalized metabolic activity of the animal. It will be many years before the many interrelationships are untangled. Nonetheless, the implications are clear. The kidney is an important organ in the *regulation* of serum phosphate levels as well as in the *conservation* of this vital ion. Perhaps quite incidentally, the excreted phosphate is the principle buffer present in urine permitting the excretion of large quantities of titratable acid at near physiological pH.

Regulation by the Intestine

The intestine, like the kidney, is an important organ in the regulation of serum levels of calcium and phosphate. Chronic disfunction in intestinal absorption, such as in sprue, can result in prolonged negative calcium balance with resulting net loss of bone substance.

A consideration of the intestinal absorption of calcium must be related to solubilities. As we have seen, normally the serum is highly supersaturated with respect to calcium and phosphate. Therefore, the transport of calcium and phosphate from the intestinal lumen into the supersaturated body fluids must require some active, cellular mechanism. Though postulated,[89, 91] such a mechanism has only recently been experimentally verified *in vitro*.[99, 105]

The technique by which this was accomplished deserves mention because it is, in effect, a model system by which the number of variables complicating an absorption study can be reduced. Intestinal segments are turned inside out, tied into sacs, and placed in oxygenated buffers *in vitro*.[17, 105, 133] By means of

radioactive calcium a net transport, *against ion-gradients,* can be demonstrated from the mucosal to the serosal side. Interestingly, this active transport mechanism seems limited to the duodenum and upper jejunum.[99, 105] This is not to say that some calcium absorption does not occur lower in the gut but the evidence here for an active mechanism is less conclusive.[17, 36, 74, 75, 128, 133] The work *in vitro* also does not provide information on the extent to which passive diffusion occurs *in vivo*. If the pH of any part of the intestine were less than 6.5,[115] it would bring the product, Ca x P_i, to a point favoring passive diffusion into the serum.[89]

The whole matter of the pH of the intestinal contents in relation to calcium absorption, however, is quite confused.[61, 62] While the preponderance of evidence favors the view that an acid-reaction or the presence of diffusible chelating agents such as citrate, favor calcium transport and that alkaline diets inhibit calcium absorption, there are many conflicting reports.[61]

There is no doubt about the importance of the normal digestion of fats. Any defect in fat absorption leads to the excretion of free fatty acids in the stool. These acids trap calcium by the formation of highly insoluble soaps and prevent its absorption.[61] Phytic acid, a constituent of cereals, has a similar action. The human lacks the enzyme phytase which in some species hydrolyzes the phosphate groups. In the intact molecule these groups strongly chelate the calcium ion and thus prevent its absorption.[62]

Experimentally it is possible to demonstrate an action similar to that of phytate by feeding large amounts of inorganic phosphate. Conversely, an extremely high Ca:P dietary ratio can inhibit the absorption of phosphate. It is somewhat ironic that these two ions, both needed for bone formation, can each inhibit the absorption of the other. Fortunately, excessively disproportionate amounts are required to show such an inhibition and in humans this can occur only on unusual dietary regimens.[61]

Balance studies indicate that calcium absorption is under hormonal regulation. Certainly, the net absorption of calcium is not related solely to dietary intake. Feeding large amounts of calcium may lead to a high fecal excretion. The amount absorbed at any dietary level seems to depend on the body's needs, being large following a negative calcium balance and low if the body is satiated.[58] Logically, such a regulation would involve the level of ionic calcium in the body fluids themselves. Since the only known hormone which is secreted in response to lowered levels of ionic calcium is parathyroid hormone,[123] it is not surprising that evidence is gathering concerning this hormone's ability to stimulate the active absorption of calcium.[16, 99, 124] Thus, in intestine as in bone, the parathyroid hormone has an action parallel to, if not synergistic with, vitamin D.

While the evidence is clear that calcium absorption is regulated, the intestinal transport of phosphate appears to be more or less passive.[58] There is, however, a tendency for the two ions to parallel each other. In other words, the active absorption of calcium from otherwise insoluble calcium phosphate salts frees soluble inorganic phosphate for ready, passive diffusion into the blood stream.

NUTRITIONAL ASPECTS

There seems little point, here, to review extensively the vast literature on the nutritional factors of special significance in the growth of bone. A number of comprehensive reviews recently have been published.[55, 61, 62, 81, 91] Of more interest here are reviews of the recent reports which place the classical physiological findings in closer relation to the newer concepts of bone formation and homeostasis already discussed.

Vitamin A

There has developed but little basic information on the metabolic activities of vitamin A in relation to bone formation since the classical observations of Mellanby.[84] Because of the high degree of resonating, conjugated unsaturation present in the vitamin A molecule, one is tempted to hypothesize that this vitamin, like others, plays some as yet unidentified role in hydrogen transport or energy conduction.[121] The only definitive evidence on this point is, of course, in the beautifully delineated case of scotopic vision.[127]

A deficiency of vitamin A in animals leads to bone deformities which, in turn, lead to nerve damage.[55, 61] No changes in bone structure, however, have so far been attributed to vitamin A deficiency in man. Some hypervitaminosis A cases, on the other hand, have been reported in the United States.[45, 69] In infants, painful swelling over the long bones and periosteal thickening of the mid-shaft (by x-ray examination) was observed. Bone fragility and spontaneous fractures are the findings in many animal species. In general, overdosage of vitamin A seems to accelerate growth and maturation of epiphyseal cartilage cells and bone resorption; with a lack of this vitamin, these processes are slowed down. The mechanisms underlying these gross and histological observations are not even surmised. In fact, it is not agreed whether osteoblasts are inhibited,[61] or osteoclasts are stimulated [134] by vitamin A. There have been implants of bone in contact with particles of vitamin A acetate in the brain.[4] Under these circumstances, (of doubtful physiological significance) massive, localized erosion by osteoclasts was seen. On the other hand, it has been found that vitamin A caused rapid resorption of bone in tissue culture without the appearance of osteoclasts.[27] Again, the physiological meaning is not clear. Especially so, since recent culture experiments with mouse calvaria demonstrated rapid osteoclastic bone resorption resulting merely from high oxygen tensions.[42, 65]

Much more information is needed before even intelligent guesses can be made regarding the effects of vitamin A on bone. In view of our newer concepts, it is easy to visualize the importance of the metabolic integrity of the cell in providing a normal matrix as a seeding site for mineralization and in dissolving the formed bone which must be removed in the remodelling associated with growth. It is also easy to visualize the possible importance of a molecule such as vitamin A, (if participating in energy-production) in maintaining metabolic integrity of cells.

Like most vitamins, despite a generalized metabolic function, vitamin A seems to show some tissue specificity, in this case acting on bone and epithelial cells.

Vitamin-B Complex

As a class, the B complex vitamins are not known for their effects on bone. Participating, as most of them do, in coenzymic functions of vital importance in energy transport and metabolic interconversions, these vitamins show important deficiency symptoms in tissues which are quicker to respond than the skeleton. Nonetheless, they are needed by practically *all* cells for normal function. It is, therefore, not surprising to find improved mineral absorption and retention upon feeding various members of the vitamin-B complex.[18] These effects are ill-defined and non-specific, however. More definitive experimentation with bone must await a greatly improved understanding of the metabolic economies of the cells participating specifically in bone growth.

Ascorbic Acid (Vitmain C)

The metabolic consequences of experimental ascorbic acid deficiency seem to be restricted to tissues of mesenchymal origin. Characteristic of this deficiency is the failure to form and to maintain the intercellular substance of the supporting structures: bone, cartilage, and fibrous connective tissue. In bone, the periosteum gradually thickens, the cells taking on the appearance of fibroblasts, and apposition of new bone ceases. Continuing resorption results in thin shafts and thin, easily fractured metaphyses and epiphyses. This frequently results in a damaged area in the cartilage, the *Trümmerfeld Zone* filled with fibrin and debris. A *Gerüstmark* or loose connective tissue, is seen in the marrow space on the shaft side of the metaphysis. Capillary penetration into the epiphyseal cartilage also fails.

Scurvy, like vitamin A deficiency, seems to differentiate between the mechanisms of calcification of epiphyseal cartilage and those of bone itself. Despite severe vitamin C deficiency, cartilage is elaborated and calcified, while osteoblasts (or what would be osteoblasts normally) fail to form osteoid.[32] In the *Gerüstmark,* collagen fibers are absent as is phosphatase, but both fibers and the enzyme quickly appear upon restoration of dietary ascorbic acid.[32]

That calcification of cartilage may differ in mechanism from that in bone itself may come as a surprise to some readers. The present discussion has stressed the catalytic role of "proper" collagen fibers in inducing crystals to form from the normally supersaturated body fluids. There is good reason to believe that this is the operative mechanism in bone. Besides the evidence already given, there exist many indications of a close association between the collagen fibers and the mineral crystals. These observations have been indirect,[15, 25, 120] such as a proof of co-orientation of fibers and crystals, as well as direct,[101, 102, 129] such as visualization by electron microscopy of the forming crystals in and on the fibers. In cartilage, however, the relationship between fibers and crystals is not clear. Growing cartilage does not contain well-formed or oriented collagen fibers.[101]

In cartilage, the crystals are laid down in "sun bursts" at a distance from the edge of the cell capsule and in no clear association with the collagen fibrils. In bone, the crystals appear associated with well-formed fibers within a fraction of a micron of the cell.[101] Thus, we are much less sure of the course of events in mineralizing cartilage than in forming bone; the differential effects of vitamin deficiencies underscore our ignorance. It is perhaps ironic that most of the research on the mechanism of calcification has been derived from studies of rachitic cartilage.

In any event, failure to mineralize osteoid which does not contain mature, normal collagen is easily understood in terms of our modern concepts. The cause of the failure of collagen formation in scurvy is less well understood, but several recent findings are very suggestive. In the first place, with adequate controls to rule out inanition, it finally has been demonstrated conclusively that "either collagen synthesis is prevented or collagen is destroyed as soon as formed" in scurvy.[47] In addition, the direct action of ascorbic acid on collagen synthesis has been demonstrated. Collagen deposition in implanted polyvinyl sponges was measured.[43] The direct injection of ascorbic acid into one of two implants in the same scorbutic animal stimulated unilateral collagen formation.

There is no question that ascorbic acid is essential for normal collagen synthesis. The question is: In what metabolic step is ascorbic acid necessary?

A distinctive feature of collagen is its high content of hydroxy amino acids, particularly hydroxyproline. Consequently, there have been recurrent suggestions that ascorbate may be necessary for the hydroxylation of proline and lysine *in vivo*. This idea is especially attractive because of the obvious oxido-reductive potentialities of the ascorbic acid molecule, a potentiality which has led to a long, but for the most part, fruitless search for some oxido-reductive function for it in energy metabolism.[98] Two lines of evidence lend support to the idea that ascorbic acid participates in hydroxylation. Of prime importance is the report that hydroxyproline and hydroxylysine are not utilized directly in collagen synthesis; rather, as shown by labelling experiments, the unhydroxylated proline and lysine of the diet are first incorporated into collagen or its precursor and subsequently hydroxylated.[100] In the second place, metal complexes of ascorbate have been shown to hydroxylate various aromatic compounds *in vitro* [122] and an ascorbate-dependent oxidase (with DPN as a co-factor), which can hydroxylate the steroid nucleus, has recently been found in the adrenals.[66] The last reference brings to mind the long-standing question concerning the association between ascorbate and the steroids of the adrenal glands.[82] The matter is still far from settled but, in terms of bone formation, the possibility of indirect effects of scurvy mediated through the adrenals must be regarded as questionable, at least for the present.[63]

Part of the reason for the persistent implication of adrenal function in scurvy may be attributed to changes found in the mucopolysaccharide componant of connective tissue. It is not certain yet whether these changes involve a direct action of ascorbic acid. The mucopolysaccharides in cartilage and wounds are usually seen to decrease in ascorbic acid deficiency accompanied by a sharp decrease

in the ratio, sulfated mucopolysaccharides : non-sulfated mucopolysaccharides.[110, 117] With the aid of radiosulfate, a decreased rate of sulfation has been observed in these tissues.[38, 65] The validity of these findings is somewhat questionable,[67] however, since most,[37] though not all,[7] of the scorbutic changes in mucopolysaccharides can be attributed to inanition.

Vitamin D

Rickets, the disease of infants and children caused by a deficiency of vitamin D, has been known for centuries. Although as early as 1918 dietary control of the disease was described,[83] the metabolic role of this vitamin is still poorly understood. The characteristic findings in vitamin D deficiency involve all three organs of calcium-phosphate homeostasis: intestine, bone, and kidney.

Longest known and best recognized is the requirement of vitamin D for the normal intestinal absorption of calcium and, indirectly therefore, of phosphate. There is general unanimity on this point and the evidence, already well-summarized,[91] is still coming in.[59] The use of inverted intestinal sacs *in vitro,* as already described, may greatly add to our understanding of the mechanisms involved. An example of future results to be expected is the report that Ca^{45} was transported from the mucosal to the serosal side of the intestine against concentration gradients and that this active transport mechanism, of limited capacity normally, was markedly impaired by D-deficiency.[105] Because, as discussed later, citrate has some important but obscure relation to vitamin D activity, studies of this ion were made concommitantly. Though citrate concentrations were higher in vitamin D treated guts, the differences could not account for observed differences in calcium transport. Some mechanism must exist in the intestine by which dietary calcium phosphates are solubilized and transported to the blood. From a physicochemical consideration, this mechanism must involve acid production or chelation or both.[89] Undoubtedly, vitamin D has an essential function in this mechanism, but its specific molecular action is not yet discernible. An "acid-mechanism," as discussed earlier, has been postulated for the transport of calcium from bone to blood.[89] Thus, it is tempting to assume that the mechanisms are similar in the two organs. This assumption may not prove to be adequate, however, since the intestinal response, unlike the bone response, has been shown to be insensitive to vitamin D dosage.[11]

Many investigators have been led to attribute the characteristic hypocalcemia of rickets to the intestinal actions of vitamin D.[62] While gut absorption may be contributory, it cannot be the primary cause. We have explained earlier that a disturbance in blood levels of calcium and phosphate can occur only if there is a disturbance in the cellular function in bone itself. Apparently, then, vitamin D is necessary for the normal functioning of bone cells. This suggestion was first made in 1935 but conclusive experimental evidence has only recently been provided. Vitamin D raises serum levels of calcium in animals receiving a diet almost devoid of calcium (0.04%).[10] By manipulation of the calcium concentration in

low calcium diets, it can even be shown that vitamin D treated rats can maintain a higher serum level of calcium than deficient rats, despite the fact that the deficient animals are absorbing more calcium.[12, 93]

Whatever the molecular events may be, because of the metabolic disturbance in osteocytes, the solubility of the mineral reservoir is diminished. This results in the characteristically low serum product, Ca x P, of rickets. Though bone crystals will not passively dissolve at these low product levels, neither can new crystallization be initiated on the newly formed matrices. At least, in model calcium phosphate-collagen systems,[27] products, Ca x P, higher than those of rickets are required for mineralization. Thus, the appearance of unmineralized cartilage and osteoid in rickets can be explained in terms of a mineral deficit. However, this does not preclude the possibility that the organic matrices also may be defective in vitamin D deficiency. No one yet knows. There is some evidence that cartilage laid down in rickets will calcify if placed in solutions of normal products, Ca x P,[71] but the situation is even less clear in the case of osteoid. Extreme lability of the nucleating power of collagen [112, 113] may result in rapid loss of calcifiability of osteoid as it remains uncalcified during the rachitic period.

With a substance such as vitamin D which can improve absorption, maintain the functions of the bone cells and thus regulate serum levels of calcium and phosphate, one might expect that direct or, at least, indirect effects on renal function would be easily observed. First reported in 1941, vitamin D does seem to have a stimulating action on tubular reabsorption of phosphate.[53] While this is believed to be a direct action,[50] the case is far from proved. An elevation of serum calcium could easily result in lowered output of parathyroid hormone which would reduce phosphate excretion. The renal action of vitamin D is muddled by bone:intestine:hormone interactions. Thus, it is not surprising that a morass of data with conclusions of every variety is being made. For example, a recent review [138] suggests that vitamin D produces hypercalcuria and is antagonistic to parathyroid hormone, despite the fact that the hormone itself produces hypercalcuria and that urinary calcium is exceedingly low in the hypoparathyroid patient.[1] Fortunately, a recent summary has been made of the many interwoven variables affecting the urinary excretion of calcium and phosphate.[116]

It is obvious that there must be some interrelation between the parathyroid hormone and vitamin D. Apparently the hormone is relatively ineffective in homeostasis when vitamin D is missing from the diet.[78] (This is more apparent with low calcium diets than with high phosphate diets.) Nevertheless, differences in action between the two agents do exist.[89] It is clear that they must act at sites in the metabolic processes which are separated yet closely related. Effects of parathyroid hormone on the production of lactate and citrate by bone cells have been described. Vitamin D, too, has an important action on citrate metabolism.[19-22, 49, 51, 52, 64, 96, 114, 125] It either augments citrate production or inhibits its utilization in several tissues.[114, 125] The citrate-effect of vitamin D has been demonstrated *in vitro* with metabolizing mitochondrial preparations.[20, 21]

The exact molecular events of vitamin D in bone growth remain to be elucidated. It is tempting to postulate that vitamin D has a role as a special co-factor or decoupler in some electron or energy transport system,[28, 135] perhaps in the DPNH:TPN transhydrogenase system.[20, 21]

In addition to the deficiency syndrome, hypervitaminosis D is frequently reported. The symptoms appear to be an overextension of the vitamin's normal functions, i.e., excessive intestinal absorption, excessive osteoclasis and bone resorption, sometimes accompanied by hypercalcemia, and hypercalcuria.

Mineral Intake

Apart from the human need of a dietary source of vitamin D, it is obvious that an adequate intake of absorbable calcium and phosphate also is necessary for normal bone growth. However, only a few recent developments in this area need be cited since many reviews of the subject have appeared over the past few years.[48, 73, 60, 136, 137]

There is a growing recognition that animals can adapt slowly to widely varying levels of calcium intake.[39, 90, 132] This observation, along with current estimations of growth requirements, suggests that even the recent downward revision of the "minimal daily requirements" may be higher than is truly necessary.[73] On the other hand, chronic tests have shown that even rats will develop a kind of osteoporosis if a diet low in calcium is fed during the early phases of life.[80] This gives substance to the clinical impression that osteoporosis may, in part, be a manifestation of long-term calcium deprivation,[95] either from inadequate intake or from faulty absorption. In any event, there are encouraging reports that excessively high calcium intakes may result in positive calcium balances in osteoporotic patients over long periods of time.[95, 130] Thus, there have developed two views: the one suggests a possible lowering of "minimal requirements," the other suggests that even present levels are too low *for some individuals*. Perhaps these two seemingly incompatible views will be resolved in terms of extreme variability of the individual requirement. In any event, new techniques employing radiocalcium [6] offer some hope for clarification of the absorption problem. Under proper conditions, it seems possible that the endogenous and exogenous sources of gastrointestinal calcium can be differentiated, thus giving the *net* dietary absorption much greater meaning.

CONCLUDING THOUGHTS

This review has concentrated on the newer findings and concepts of bone formation and nutrition. It is probable that not all of the concepts presented are generally accepted by experts in the various specialized fields of study that have been discussed. Accepted or not, some of the concepts are undoubtedly incorrect in detail if not in substance. Nonetheless, it was the purpose of this biochemical survey to present the established facts and working hypotheses relating nutrition and bone formation in the hope of developing an interaction between the two.

It has, at least, exposed the areas of ignorance, the few areas where critical work is clearly indicated and one area where research is underway. Ignorance of the relative quantitative contributions of the intestine, bone and kidney to calcium-phosphorus homeostasis and the complications always present in the *in vivo* system make the accurate determination of a minimum daily requirement impossible at present. The molecular function of vitamin A, though well defined in vision, is a mystery in any other part of the body in spite of the frequent, though conflicting, reports (omitted here) of changes in various enzyme systems. A better documentation of these changes is required before the role of vitamin A in bone can even be indicated. The case of the vitamin-B complex is quite the other way. In spite of a fine delineation of its cellular function in soft tissues, the metabolic affairs of bone cells are too poorly understood to make use of this knowledge. As for ascorbic acid, however, promising investigations of both bone and nutrition, have developed to the point where the action of this vitamin on bone formation is bound to progress. There are promising indications that ascorbate is essential in the hydroxylation of proline and lysine in the collagen molecule and thus in the preparation of the sites of mineral nucleation. Still much more work is needed to establish the principles involved, not to mention the details. The traditional view that vitamin D stimulates bone formation is under critical attack. The new view ascribes the apparent osteogenic action of vitamin D to its indirect effect of mobilizing for blood the calcium and phosphorus in the mineral reservoir of bone and in the intestine via a metabolic role in acid production. While investigators are now at least looking in the right place, metabolically speaking, the molecular events are not yet clearly seen.

Metabolic bone disease has not been mentioned. It is all too obvious that much more work is needed on the cyclic, interdependent, homeostatic mechanisms in the intestine-bone-kidney system before rational therapy can be developed.

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